Avian Semen Cryopreservation: What Are the Biological Challenges?[1]

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ABSTRACT The value of the ability to cryopreserve and store germplasm has long been recognized for indefinite preservation of genetic material, especially for at-risk populations. In contrast to domestic livestock species, cryogenic storage of poultry semen is not reliable enough for germplasm preservation. The relatively low fertilizing ability of frozen/thawed poultry sperm most likely results from physiological sensitivity to the cryogenic process coupled with the requirement for prolonged sperm functionality in the hen reproductive tract; however, the concept of defining these physiological challenges has been underemphasized. For example, alterations in membrane carbohydrate content and diminished energy production in frozen/thawed sperm have important implications for successful gamete interaction. Recent data suggests that both glycoconjugate content and adenosine triphosphate (ATP) generation are affected by cryopreservation. Moreover, susceptibility to the cryogenic process seems to vary among lines and strains of birds, as illustrated by line-specific differences in ATP concentrations of frozen/thawed sperm from pedigreed commercial layers. Research based on biochemical and molecular comparisons of sperm among lines may lead to identification of factors that influence the freezability of poultry semen.

Key words: cryopreservation, semen, fertility, poultry

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INTRODUCTION

More than 50 yr ago, the discovery of glycerol's cryoprotective properties pioneered the success of modern cryobiology and led to the development of semen cryopreservation for a wide range of species. Despite the fact that this scientific breakthrough was accomplished with rooster semen (Polge, 1951), the overall fertility rates with frozen/thawed poultry semen are highly variable and not reliable enough for use in commercial production or preservation of genetic stocks. Moreover, significant differences exist among the commercial poultry species (turkey, broiler-type chicken, and layer-type chicken) in terms of the viability and functionality of sperm after cryopreservation. In particular, the fertility rates from frozen/thawed turkey semen consistently have been lower than cryopreserved chicken semen (Nelson et al., 1980; Sexton, 1981; Kurbatov et al., 1986; Schramm and Hubner, 1988; Wishart, 1989). Several comprehensive reviews have been published summarizing the empirical studies involving cryoprotectant type and packaging method, as well as freezing and thawing rates, for avian sperm cryopreservation (Lake, 1986; Hammerstedt and Graham, 1992; Donoghue and Wishart, 2000).

The purpose of this invited paper is to highlight the biological challenges associated with avian semen cryopreservation and to suggest novel approaches for investigating the viability of frozen/thawed poultry semen.

BIOLOGICAL CHALLENGES: A SPECIES COMPARISON

The greatest progress in commercializing semen preservation has been achieved by the dairy and beef cattle industries, where semen cryopreservation has been optimized, standardized, and automated. Although this level of success with bull semen has not been achieved with other livestock species, such as pigs or sheep (Holt, 2000), the fertility rates of cryopreserved poultry sperm are dramatically lower than any of the domestic mammalian species. It has been estimated that cryopreserved rooster semen retains <2% of the fertilizing ability of fresh semen (Wishart, 1985). A closer look at the differences in bovine and poultry reproductive strategies provides the basis for the dichotomy in the fertilizing ability of cryopreserved sperm from these species.

From a broad perspective, relatively few viable bovine sperm are required for successful fertilization, as most females ovulate a single ovum during the estrous cycle. In contrast, poultry hens are managed to produce eggs daily for either a 5- to 7-mo period (turkey hen) or a

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12- to 14-mo period (chicken layer hen). Further, bovine artificial insemination is generally timed to coincide within 24 to 48 h of ovulation; whereas, hens are inseminated once per week during the egg production period. Weekly insemination requires that a relatively high number of poultry sperm remain viable for a minimum of 7 d within the hen's sperm storage tubules (**SST**) to ensure fertilization between inseminations. Even with fresh, unstored semen, only 1 to 2% of inseminated sperm reach the SST (Bakst et al., 1994); therefore, it is likely that any sperm damage from a cryogenic cycle could severely affect the numbers being stored in the SST. Comparatively, this requirement for prolonged sperm functionality means that there is a much lower tolerance for poor sperm survival after cryopreservation of poultry semen compared with bovine semen.

Another major difference between bovine and poultry reproductive biology that affects semen cryopreservation is the semen volume obtained per collection. The average ejaculate volume of a bull ranges from 5 to 8 mL; whereas semen volumes collected from poultry are considerably less, ranging from 0.1 to 0.3 mL. Although it is true that the average sperm concentration of poultry semen is much higher (6 to 10×10^9 sperm/mL) than bull semen (1 to 2 $\times 10^6$ sperm/mL), poultry sperm function is adversely impacted by excessive dilution (Sexton, 1977; Duplaix and Sexton, 1983; Bakst, 1990). This 'dilution effect' may lower the overall fertilizing ability of frozen/thawed poultry semen.

Narrowing the scope of the discussion from basic reproductive differences to comparative sperm physiology provides several characteristics unique to poultry that likely influence the outcome of semen cryopreservation. One notable difference is the morphology of the avian sperm cell compared with the bovine spermatozoan. The overall filiform shape of the avian spermatozoan confers a smaller surface area-to-volume ratio and a more condensed nucleus than the paddle-shaped bovine sperm cell. These morphological characteristics explain some of the differing responses to constraints imposed by the cryopreservation process, such as the critical osmolality. For rooster spermatozoa, the critical osmolality, or the osmolality at which 50% of the sperm cells are lysed, is lower (17 mOsm) than for bull spermatozoa (36 mOsm), indicating that poultry sperm have a smaller capacity for increased volume than bull sperm (Watson et al., 1992). In fact, poultry sperm appear to be highly susceptible to morphological disruptions during the freeze/thaw process. Frozen/thawed rooster sperm had higher incidences of ultrastructural abnormalities of the mitochondria, midpiece, and perforatorium than fresh sperm (Xia et al., 1988), and electron microscopy also revealed that 60% of turkey sperm organelles suffered irreversible damage after cryopreservation (Bakst and Sexton, 1979), notably visible as swollen midpieces (Marquez and Ogasawara, 1977). Comparatively, avian sperm are longer (80 to 90 μm) than bull sperm (50 to 60 μm), and the more compact morphology of bovine sperm makes them less susceptible to injury from mechanical manipulations, such as pipetting and centrifugation, common during semen cryopreservation (Agca and Critser, 2002). The fact that the avian sperm tail is approximately 8 times the length of the sperm head also predisposes poultry sperm to be more sensitive to freezing damage (Donoghue and Wishart, 2000).

The process of cryopreservation imposes numerous stresses on not only the physical features of sperm, but also the chemical components essential for functions such as energy metabolism to support motility. Both poultry and bovine spermatozoa have a greater inability to maintain adenosine triphosphate (**ATP**) content after cryopreservation (Wishart and Palmer, 1986; Soderquist et al., 1991). Moreover, species-specific differences in metabolism are likely to contribute to the differences in the ability of turkey and chicken spermatozoa to survive the freeze/thaw process. Although chicken sperm are equally capable of metabolism in aerobic or anaerobic environments (Sexton, 1974), it is well established that turkey spermatozoa provide energy for metabolic requirements by oxidative respiration rather than glycolysis (Sexton, 1974; Wishart, 1981; Sexton and Giesen, 1982). Further, turkey and chicken sperm respond differently when subjected to lower temperatures. The fertilizing ability of turkey sperm was compromised as soon as temperatures dropped below 15°C; whereas equilibrium at 5°C did not reduce fertilizing ability of rooster semen (Sexton, 1981).

The ability of sperm to survive cryopreservation and remain functional requires methodologies that are within the biophysical and biological limits defined by the cryobiological characteristics of each species (Agca and Critser, 2002). It is evident that successful cryopreservation methods for turkey and chicken sperm are likely to require different strategies. This rationale also can be extended for the many unique poultry research stocks that need to be preserved, as the tolerance of poultry sperm to cryopreservation varies among genotypic strains of chickens (Bacon et al., 1986; Froman and Bernier, 1987; Tajima et al., 1990; Alexander et al., 1993). With respect to the numerous poultry stocks currently at risk, it is unfortunate that a broiler-type line selected for the duration of fertility of frozen/thawed semen (Ansah and Buckland, 1983) no longer exists. After 8 generations of selection, both physiological changes and biochemical differences were detected between the lines selected for resistance to cryo-injury and the control lines. For example, sperm from the cryo-resistant line had lower levels of cholesterol within the plasma membrane and were more permeable to glycerol than the control line (Ansah and Buckland, 1982). Sperm from the selected line also exhibited higher oxygen uptake, indicating that intracellular organelles were more resistant to freeze/thaw injury (Scott et al., 1980). It also was suggested that a particular protein found in seminal plasma of males from the selected line might have contributed to increased cryoprotectant permeability (Bentley et al., 1984).

NEW APPROACHES: IDENTIFYING COMPROMISED SPERM FUNCTION

The existence of quantitative differences between species and even among lines/strains is an important deter-

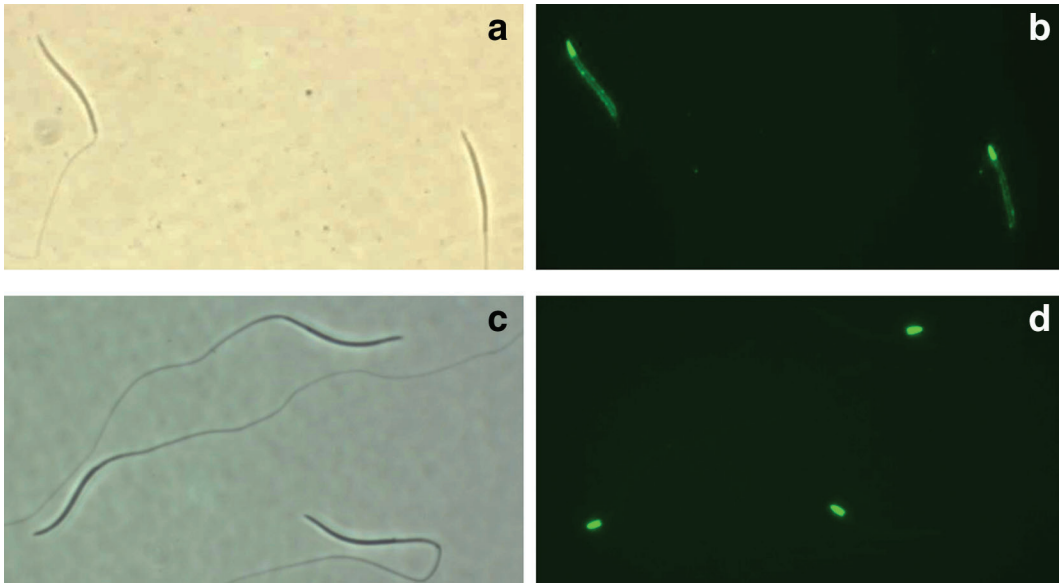

Figure 1. Phase constrast (a, c) and fluorescent (b, d) images of fresh poultry spermatozoa incubated with fluorescein isothiocyanate (FITC)-labeled GS-1, a lectin which specifically binds α-galactose residues. GS-1 binding patterns differed ($P < 0.05$) between turkey (a, b) and rooster (c, d) spermatozoa, and indicated that α-galactose residues were present on the acrosome region of both species but not the head region of rooster spermatozoa.

minant of the fertility of cryopreserved semen and necessitates that the development of successful freezing procedures involves more than the identification or application of novel cryoprotectants and additives (Holt, 2000). It has been shown that, although turkey semen cryogenic protocols yield 80% motile sperm with intact plasma membranes, frozen/thawed turkey sperm retain only 30% of their binding ability (Hammerstedt and Shultz, 1994). These data are suggestive that the essential sperm functions being disrupted by current cryogenic protocols involve compromises to the plasma membrane more subtle than lysis.

The sperm glycocalyx is a dense carbohydrate layer extending 20 to 60 nm from the cell surface (Bearer and Friend, 1990) that emanates from either plasma membrane proteins (glycoproteins) or lipids (glycolipids). The sperm glycocalyx is modified extensively during sperm transport and maturation and represents the primary interface between the male gamete and its environment. Critical glycoconjugates for poultry gamete interaction include sialic acid, which has been implicated for both sperm passage through the vagina (Steele and Wishart, 1996) and sperm sequestration in the hen's SST (Froman and Thursam, 1994), and N-acetyl-D-glucosamine, which is necessary for sperm/egg interaction (Robertson et al., 2000).

Table 1. Adenosine triphosphate concentrations (pmol/10^9 sperm cells) in fresh and frozen/thawed rooster spermatozoa

Male	Fresh sperm	Frozen/thawed sperm
1	5,163.2 ± 0.34[a]	65.04 ± 0.02[b]
2	3,644.20 ± 0.53[a]	58.97 ± 0.06[b]
3	2,141.50 ± 0.45[a]	64.21 ± 0.03[b]
4	3,321.70 ± 0.3[a]	67.03 ± 0.03[b]
5	2,958.12 ± 0.67[a]	59.61 ± 0.04[b]
6	2,772.34 ± 0.55[a]	50.26 ± 0.03[b]
7	1,643.71 ± 0.32[a]	50.49 ± 0.05[b]
8	1,434.16 ± 0.67[a]	48.05 ± 0.02[b]
9	2,734.49 ± 0.23[a]	52.07 ± 0.04[b]
10	3,600.65 ± 0.44[a]	59.01 ± 0.03[b]
11	1,842.32 ± 0.62[a]	63.29 ± 0.07[b]
12	3,194.77 ± 0.14[a]	60.07 ± 0.05[b]

[a,b]Different superscripts indicate significant differences within rows.

It seems plausible that glycoproteins critical for sperm function are compromised during the freeze/thaw cycle; however, little is known about the composition and spatial distribution of the surface glycoconjugates of poultry sperm. We have initiated characterization of the poultry sperm glycocalyx using fluorescein isothiocyanate-conjugated lectins specific for each of the known carbohydrate residues (Peláez and Long, 2005). As expected, sialic acid residues were distributed along the entire cell surface of both turkey and chicken spermatozoa. Other carbohydrate residues were segregated among specific morphological zones. For example, α-mannose and α-glucose were detected only in the plasma membrane overlying the head region; whereas, N-acetylglucosamine residues were distributed mainly along the acrosome region. One notable difference between turkey and chicken sperm was the absence of α-galactose residues on the head region of chicken sperm (Figure 1). With the characterization of carbohydrate residues on the surface of poultry sperm complete, current studies are focused on elucidating alterations of the glycocalyx in frozen/thawed sperm.

Motility is another essential sperm function that is compromised as a result of poultry semen cryopreservation; 30 to 60% reductions occur after freeze/thaw cycle (Westfall and Harris, 1975; Bakst and Sexton, 1979; Scott et al., 1980; Wishart and Palmer, 1986). The chemical energy required for sperm motility is supplied by the mitochon-

Table 2. Post-thaw assessment of semen samples from 4 pedigreed commercial lines[1]

Line	ATP concentration (pmol/10^9 sperm)	Membrane-intact sperm (%)	Fertile eggs (%)	Hatched chicks (%)
1	106.8 ± 0.83	44.3 ± 0.34	18.6 ± 1.6	98 ± 1.0
2	62.4 ± 0.97	40.7 ± 0.51	14.3 ± 5.2	96 ± 1.2
3	118.8 ± 0.62	55.2 ± 0.23	20.2 ± 3.4	100
4	124 ± 0.43	53.6 ± 0.25	28.3 ± 4.5	100

[1]n = 10 males per line.

dria in the form of ATP. Given the apparent sensitivity of poultry sperm mitochondria to the freeze/thaw process, it is not surprising that ATP production would be compromised after cryopreservation. Currently, we are investigating the ATP production of stored poultry sperm using a combination of assays designed to identify specific pathways that may be disrupted, such as enzyme inactivation and ion flux. One limitation in measuring ATP concentration has been removed by the development of a highly repeatable assay that permits simultaneous measurement in multiple samples (Long and Guthrie, accepted). Key features of this assay include addition of a phosphatase inhibition step, specific processing conditions for samples stored at –20°C, and use of a plate reader equipped with a luminescence detection mode. Using this assay, we have determined that the extent of ATP loss in rooster sperm after cryopreservation is severe (Table 1). Interestingly, although perhaps not surprisingly, ATP concentrations in frozen/thawed sperm vary among commercial and research layer lines, as well as commercial broiler lines (Long, unpublished data).

Accumulating evidence suggests that there is significant variability among poultry species and lines, as well as individual males, with respect to sperm fertilizing ability following a freeze/thaw cycle. It follows, then, that biochemical and molecular-biological comparisons of sperm among lines may lead to identification of factors that influence the freezability of poultry semen (Tajima et al., 1990). One approach we are taking is to determine the freezability of semen from individual roosters by cryopreserving samples from multiple males within multiple lines. Preliminary data from 4 pedigreed commercial layer lines demonstrates the feasibility of this model for understanding the compromised physiology of cryopreserved spermatozoa (Table 2). As part of an ongoing project, semen samples are being frozen from individual roosters (20 males per line) at 6 and 12 mo of age. Analysis of these thawed semen samples is providing the basis for new approaches to cryopreservation of poultry sperm.

A final important consideration is the immediate need for preservation of semen from poultry research stocks currently at risk. Developing alternative cryopreservation protocols for poultry semen will be a lengthy process, and many research stocks may disappear in the interim. To provide a short-term solution for this problem, we developed a means of post-thaw processing to improve the fertility of poultry semen frozen with glycerol as the cryoprotectant (Long and Kulkarni, 2004). Although glycerol is the cryoprotectant of choice for poultry sperm, this cryoprotectant has been repeatedly demonstrated to be contraceptive in the hen at concentrations >0.1 *M* (Hammerstedt and Graham, 1992). Reduction of the glycerol concentration in thawed poultry semen improves fertility; however, this approach is only valid for critical research stocks that may be lost within the near future. Research efforts to develop alternative cryopreservation protocols are necessary, especially in the context of the emerging biological differences among poultry varieties.

SUMMARY

Recent research promises new areas for improving the success of poultry semen cryopreservation. During the past several decades, an overlooked concept has been the importance of defining the physiological challenges associated with freezing poultry semen. Elucidation of the cellular and molecular changes that occur during cryopreservation is key for developing strategies to circumvent these challenges, as opposed to the largely empirical efforts to date. It is also important to consider the effects of line or strain differences and to realize that methods may need to be adjusted accordingly. Even with such adjustments, the biological competence of cryopreserved samples should be determined to ensure the best possible outcome, especially in the context of germ-line retrieval. Finally, it should be noted that the high fertility rates desired for commercial production (>96%) are not mandatory for the success of poultry sperm cryopreservation. Germ-line retrieval is feasible with modest fertility rates (60 to 70%) as long as the hatchability of fertile eggs remains high.

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